Study by ^{23}Na-NMR, ^{1}H-NMR, and Ultraviolet Spectroscopy of the Thermal Stability of an 11-Basepair Oligonucleotide

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ABSTRACT ^{23}Na-NMR, ^{1}H-NMR, and ultraviolet (UV) spectroscopy have been used to study the thermal stability of the double helix structure of an 11-basepair oligonucleotide. The denaturation curves obtained by ^{23}Na-NMR and UV are analyzed using a two-state model. The melting temperature and ΔH^0 obtained are identical within experimental error, suggesting that modifications in the ionic atmosphere, probed by ^{23}Na-NMR, and the modifications in the basepair stacking, probed by UV, occur at the same temperature. Additional dynamical information on the denaturation process has been obtained by ^{1}H-NMR: slow exchange is observed between the thymine methyl resonances, and the disappearance of imino protons shows that a single basepair opening does not contribute significantly to proton exchange.

INTRODUCTION

Novel drugs, which can be small molecules, proteins, or oligonucleotides, are currently being designed to target nucleic acids. In order to develop this new medicinal strategy in a rational way, there is a need for information regarding the structure, stability, and dynamics of nucleic acids in the presence or in the absence of these drugs.

The structure of nucleic acids can be obtained from x-ray and NMR data. NMR has the advantage of studying systems in solution and being able to yield information, via relaxation rates and NOE measurements, on the dynamics of oligonucleotides (Forster and Lane, 1990; Searle and Lane, 1992; Borer et al., 1994; Lane, 1995). Unfortunately, only small systems can be studied and the largest DNA duplex structure obtained by NMR deposited to date in the Nucleic Acid Data Base corresponds to a non-self-complementary double-strand 13-basepair oligonucleotide (Mujeeb et al., 1993).

Regarding the stability of the double-strand structure of nucleic acids and the influence of drugs on this stability, information is most commonly obtained from thermal denaturation experiments. The denaturation curve reflects the double helix to single-strand equilibrium and is generally analyzed using an "all-or-none," also called "two-state," model. In this model, each nucleic acid is considered to be either totally in the double helix form or totally dissociated. A melting temperature (T_m), defined as the temperature at which half of the nucleic acids are in the single-strand form, can be extracted from the experimental data. The two-state model is usually regarded as valid for short oligonucleotides (Albergo et al., 1981). However, for larger systems, and even in certain cases for short oligonucleotides, denaturation can be more complex. For instance, the partial unpairing of the double helix and the presence of unfolding intermediates have been reported (Cantor and Schimmel, 1980; Hopkins et al., 1993; SantaLucia et al., 1996). The melting process is generally followed by ultraviolet (UV) spectroscopy but can also be studied by other techniques, such as ^{1}H- and ^{31}P-NMR (Patel and Canuel, 1979; Patel et al., 1982a; Petersheim and Turner, 1983b; Roongta et al., 1990). When individual resonances corresponding to specific nucleotides can be monitored, NMR has the advantage of offering the possibility to verify the validity of the two-state model (Patel et al., 1982a; Petersheim and Turner, 1983b).

Nucleic acids are highly charged polymers and it is important to study the influence of their counterion atmosphere on the behavior of the nucleic acids. Techniques that probe the nucleic acid bases, such as UV spectroscopy, circular dichroism, and ^{1}H-NMR, have been extensively used to study the influence of the ionic atmosphere on the structure of nucleic acids (Hanlon et al., 1978; Xu et al., 1993b; Rouzina and Bloomfield, 1998), on the thermal denaturation of nucleic acids (Record, 1975; Patel et al., 1982b; Williams et al., 1989) and also on the binding of ligands (Record et al., 1976, 1981). Insight into these issues can also be obtained by probing the system via the counterion atmosphere. For example, ^{23}Na-NMR experiments have been used to study the interaction mode of different charged drugs with DNA (Mariam and Wilson, 1983; Dinesen et al., 1989; Eggert et al., 1989; Padmanabhan et al., 1991; Hald and Jacobsen, 1992; Casu et al., 1996, 1997) and the thermal denaturation of DNA (Bleam et al., 1983; Mariam and Wilson, 1983; Van Dijk et al., 1987; Lematre et al., 1988; Groot et al., 1994). Cation NMR experiments have also provided information on the ion distribution around the nucleic acids (Reuben et al., 1975; Bleam et al., 1983; Braunlin, 1995; Deng and Braunlin, 1996), the structure and sequence-specific recognition of cations (Nordenskiöld et al., 1984; Hud et al., 1998, 1999), the relative binding affinities of monovalent cations for nucleic acids (Anderson

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et al., 1978; Bleam et al., 1980), and the axial and radial translational diffusion of cations relative to the helix axis (Halle et al., 1984; Van Dijk et al., 1987; Groot et al., 1994).

Generally, in NMR studies it seems advantageous to determine molecular structural parameters via studies of atoms that constitute the molecule (^{13}C, ^{15}N, ^{31}P, or ^{1}H). In the case of nucleic acids, these "classical" NMR spectroscopies are, however, not as powerful as they are for the study of polypeptides and proteins. Moreover, they are unable to provide much information on the ionic atmosphere in the vicinity of the oligonucleotide. Under these conditions, probing the system via the counterions appears as a very interesting complementary technique. The counterions can be considered as spins-spies that probe the DNA.

It is apparent from the above introduction that many different experimental techniques have been used to study the thermal denaturation of nucleic acids. Comparison of the results obtained from the different techniques is important because it is not clear whether the techniques that probe the environment of the nucleic acid bases (UV, ^{1}H-NMR, CD) and ^{23}Na-NMR, which probes the nucleic acid via its counterion atmosphere, are sensitive to the same aspects of the denaturation process. Indeed, when ^{23}Na-NMR and UV melting experiments have been performed on the same DNA systems, the denaturations probed by UV have been reported to occur sometimes at the same temperatures (Mariam and Wilson, 1983; Van Dijk et al., 1987) and sometimes at higher temperatures than the denaturations probed by ^{23}Na-NMR (Bleam et al., 1983; Lematre et al., 1988). These discrepancies show the need for a more quantitative comparison of the data obtained from ^{23}Na-NMR and from UV spectroscopy. Because natural DNA used in most ^{23}Na-NMR denaturation studies is rarely a pure system and varies according to its origin, it is difficult to compare results reported in the literature by different groups. In this paper we report results obtained on the denaturation of an 11-basepair synthetic oligonucleotide. The chosen oligonucleotide d(C^1 G^2 C^3 A^4 C^5 A^6 C^7 A^8 C^9 G^{10} C^{11}) · d(G^{12} C^{13} G^{14} T^{15} G^{16} T^{17} G^{18} T^{19} G^{20} C^{21} G^{22}) has already been the subject of structural studies (Jourdan, 1998) and is a well-defined system for which the denaturation process is certainly easier to describe than for DNA. Extensive study of its thermal denaturation by ^{23}Na-NMR, ^{1}H-NMR, and UV spectroscopy and the comparison of the results are reported.

MATERIALS AND METHODS

Preparation of samples

The two complementary strands of oligonucleotide were purchased in the sodium salt form from Eurogentec (Liege, Belgium). Each strand was desalted on inverse phase C-18 columns (Sep-Pak cartridges) by elution with solutions containing increasing amounts of acetonitrile in water. Pairing of the strands was achieved by heating a water solution containing an equal number of moles of each strand to 353 K for 10 min and then letting the system cool slowly. The oligonucleotide concentrations, expressed in terms of phosphate concentration, were determined spectrophotometrically by measuring the absorbance at 260 nm and using $\epsilon = 9960$ M^{-1} cm^{-1} for the strand d(CGCACACACGC), called strand A; $\epsilon = 10130$ M^{-1} cm^{-1} for the strand d(GCGTGTGTGCG), called strand T; and $\epsilon = 8408$ M^{-1} cm^{-1} for the double-strand oligonucleotide. The sodium concentration of the oligonucleotides samples were measured by ^{23}Na-NMR by comparing the signal in the solution to the signal from a reference solution placed in a concentric 10-mm tube (NaCl at known concentration with 60 mM of the chemical shift reagent $DyCl_3$). The pH in the double-strand oligonucleotide sample was 8.5 ± 0.1. The desalted DNA sample was provided by the group of A. Lai at the university of Cagliari. Its preparation is described elsewhere (Casu et al., 1996).

UV melting experiments

The absorbance versus temperature curve was measured at 290 nm on a Perkin-Elmer lambda 40 spectrophotometer equipped with a PTP-1 DNA melting kit and using a cell with a 2-mm pathlength. The heating rate was 0.5 K/min. The temperature was measured directly in the sample by a resistance adapted on the cap of the cell.

NMR experiments

NMR samples were prepared in deionized H_2O + 10% D_2O. ^{1}H-NMR experiments were performed on a Varian Unity 600 MHz spectrometer using a jump-return pulse sequence (Sklenar and Bax, 1987). ^{23}Na-NMR experiments were performed on a Bruker 250 MHz spectrometer, a Bruker 360 MHz spectrometer, and a Varian Unity 600 MHz spectrometer. Longitudinal relaxation rates were measured using the inversion-recovery sequence. Transverse relaxation rates were measured using the Carr-Purcell-Meiboom-Gill (CPMG) (Carr and Purcell, 1954; Meiboom and Gill, 1959) sequence with echo delays of 2 ms or using the spin-lock sequence (Leipert et al., 1975). For all relaxation rates measurements at least 13 different τ delays were used. The transverse relaxation rates obtained by CPMG and spin-lock were checked by comparison with the linewidth obtained from lineshape deconvolution. Triple quantum filtered (TQF) signals were recorded using the standard sequences with a mixing time of 3 μs and an evolution time of 20–30 ms (Jaccard et al., 1986).

Temperature calibration

For the comparison of the ^{23}Na relaxation rates on the three spectrometers at a precise temperature (299 K) the displayed temperatures were adjusted to obtain the same difference between the chemical shifts of the hydroxyl and aliphatic protons of an ethylene glycol sample contained in a sealed tube (Van Geet, 1968). For the comparison of the melting experiments performed by ^{23}Na-NMR, ^{1}H-NMR, and UV, the displayed temperatures on the Bruker 360 MHz, Varian 600 MHz, and Perkin-Elmer-UV spectrometers were calibrated using the melting point method (Piccinni-Leopardi et al., 1976) with samples of undecanoic acid (mp = 300–301.5 K), myristic acid (mp = 326–327 K), azobenzol (mp = 341 K), and benzil (mp = 368 K). The accuracy of temperature measurements was ±1 K.

RESULTS AND DISCUSSION

^{23}Na-NMR

Among the most common biologically occurring monoatomic cations (Na^+, K^+, Mg^{2+}, Ca^{2+}), ^{23}Na is the easiest to observe by NMR (100% natural abundance and a resonance frequency close to the resonance frequency of ^{13}C).

The ^{23}Na nucleus possesses a spin quantum number of 3/2 and its magnetic relaxation is dominated by the quadrupolar mechanism that results from the interaction between the nuclear quadrupole moment and fluctuating electric field gradients. Because sodium is monoatomic, these electric field gradients are of intermolecular origin. The ^{23}Na relaxation rates are therefore very sensitive to the Na^+ environment, and ^{23}Na can be considered as a spin-spy. ^{23}Na-NMR relaxation experiments should provide information about the stability of double-strand oligonucleotide because the ^{23}Na environment *is* different if the oligonucleotide is in the double- or the single-strand form (Van Dijk et al., 1987). Furthermore, if the relaxation of ^{23}Na is outside the extreme narrowing condition ($(\omega_0 \cdot \tau_c)^2 \ll 1$ where ω_0 is the resonance frequency of the nucleus and τ_c is the correlation time of the fluctuating electric field gradients), it *may* also be possible to obtain dynamical and structural information on the interaction between the ^{23}Na ion and its environment. Indeed, outside the extreme narrowing condition, the longitudinal and transverse relaxation of ^{23}Na are characterized by two relaxation rates (R_{1f} and R_{1s}; R_{2f} and R_{2s}) (Hubbard, 1970; Bull, 1972) from which, in favorable cases, it may be possible to extract amplitudes and correlation times characterizing the interactions between the quadrupolar moment of the ^{23}Na and the fluctuating electric field gradients. It has been mentioned in the literature that the extreme narrowing condition is always obtained for ^{23}Na in oligomeric DNA solutions, but no mention is made of a size limit (Xu et al., 1993a). The section entitled "Characterization of ^{23}Na relaxation" deals with this aspect of our ^{23}Na-NMR study of the 11-basepair oligonucleotide.

In polyelectrolyte solutions the ^{23}Na relaxation rates are usually interpreted on the basis of a two-environment model: the sodium ions are either in the bulk (B), where the relaxation is considered essentially not affected by the presence of the polyelectrolyte, or in the vicinity of the polyelectrolyte (A). In DNA solutions the exchange between these two environments is fast on the NMR timescale and the observed relaxation rate is the weighted sum of the relaxation rates in the bulk and in the vicinity of the DNA:

$$R_i = x_A \cdot R_{i,A} + (1 - x_A) \cdot R_{i,B} \quad (1)$$

where x_A is the fraction of ^{23}Na in the vicinity of the nucleic acid and R_i is a longitudinal or transverse relaxation rate.

Regarding x_A, Manning's counterion-condensation theory (Manning, 1978) states that if the linear charge density parameter of a polyion (ξ) exceeds 1, then the fraction of bound cations per phosphate group is equal to $1 - \xi^{-1}$ (ξ = 4.2 for double-strand DNA). This theory is applicable for long polyions and a large range of ion concentrations (from infinite dilution to 1 M for double-strand DNA). The number of ions in the vicinity of these polyions is therefore independent of the total ion concentration. Consequently, addition of excess salt in the polyion solution will simply increase the number of ions in the bulk. This results in a decrease in x_A and therefore in the weight of $R_{i,A}$ in the observed relaxation rate (Eq. 1). In order to maximize the proportion of counterions in the vicinity of the polyion it is therefore important to work at low sodium concentrations. In the present study, the two strands of the oligonucleotide were conditioned so that Na^+ was the only counterion, and so that the Na^+-to-phosphate ratio was reduced to a value close to 1 in the single- and double-strand oligonucleotide (experimental value of 1.1). For oligoions it is likely that the number of sodium ions in the vicinity the oligonucleotide is not totally independent of the total sodium and oligonucleotide concentrations. However, the decrease in the number of Na^+ ions upon desalting seems to be small at the high oligonucleotide concentration used for NMR (Fenley et al., 1990; Braunlin, 1995; Stein et al., 1995).

Conformational changes that modify the linear charge density, such as denaturation, are expected to modify the fraction of cations in the vicinity of the polyelectrolyte (x_A in Eq. 1). Furthermore, since ^{23}Na relaxation rates depend on the electric field gradient fluctuations, modifications in the linear charge density will also influence the relaxation rates of the ^{23}Na in the vicinity of the polyelectrolyte ($R_{i,A}$ in Eq. 1). A variation in the observed relaxation rate is therefore expected upon denaturation as a consequence of the modification of x_A and/or $R_{i,A}$. The ^{23}Na relaxation study of the thermal denaturation of the 11-basepair oligonucleotide is covered in the section entitled "^{23}Na-NMR study of oligomeric DNA duplex denaturation."

Characterization of ^{23}Na relaxation

Results reported in the literature for ^{23}Na outside the extreme narrowing conditions in polyelectrolyte solutions (Levij et al., 1981; Braunlin, 1995) show that it is difficult to extract both longitudinal relaxation rates from the inversion-recovery experiments, but that both transverse relaxation rates (R_{2s}, R_{2f}) can be determined from lineshape analysis, CPMG experiments, or TQF experiments (a signal is observed in a TQF experiment only if the nucleus is outside the extreme narrowing conditions (Jaccard et al., 1986)). These experiments were performed at 299 K with a 360 MHz spectrometer (^{23}Na resonance frequency of 95.3 MHz), on a 0.4 mM solution (7.6 mM in phosphate) of the 11-basepair oligonucleotide and, for comparison, on a 4.5 mM NaCl solution and on a 3.3 mM (in phosphate) solution of sonicated calf thymus DNA. A TQF signal is observed for the DNA solution (Fig. 1 *a*) but not for the oligonucleotide and NaCl solutions. Two Lorentzians are needed to fit the ^{23}Na lineshape in the DNA solution (Fig. 1 *b*), while a single Lorentzian is sufficient for the oligonucleotide (Fig. 1 *c*) and the NaCl samples. Using a single exponential analysis of the inversion-recovery and CPMG experiments,

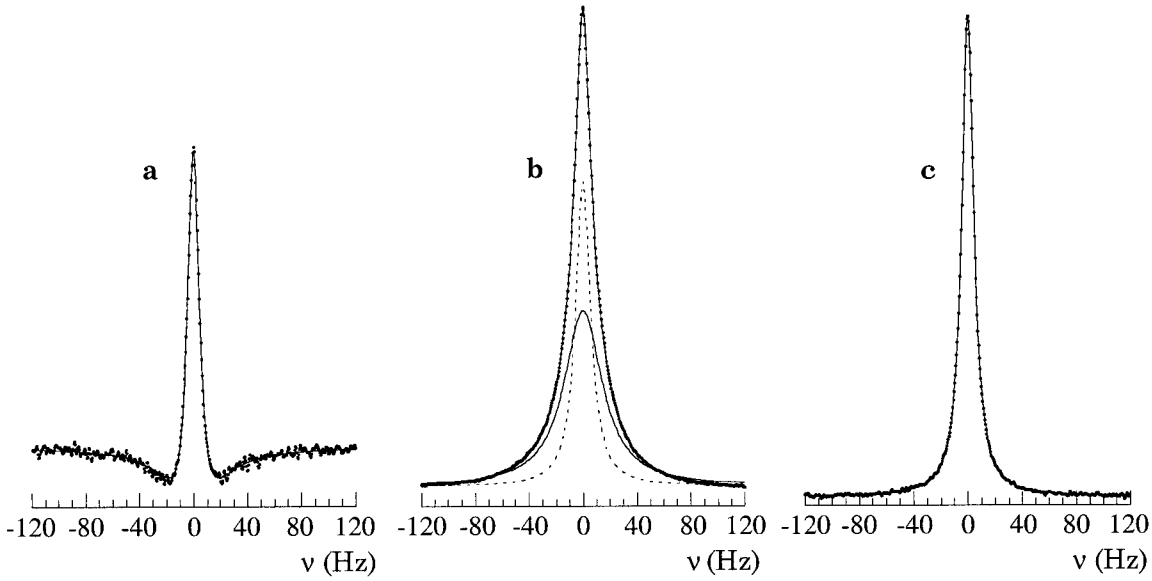

FIGURE 1 (*a*) ^{23}Na-NMR TQF signal in a sonicated solution of 3.3 mM in phosphate calf thymus DNA ($[Na^+]/[P] = 1.4$); (*b*) ^{23}Na-NMR signal in the same solution as in (*a*) and the experimental signal is decomposed into a broad Lorentzian (*solid line*) and a narrow Lorentzian (*dashed line*); (*c*) ^{23}Na-NMR signal in a 0.4 mM (in oligonucleotide) solution of the double-strand 11-basepair oligonucleotide ($[Na^+]/[P] = 1.1$).

R_1 is found to be slightly smaller than R_2 in the oligonucleotide sample ($R_1 = 28.1 \pm 0.6\ s^{-1}$ and $R_2 = 32.7 \pm 1\ s^{-1}$), while both relaxation rates are identical in the NaCl sample ($R_1 = R_2 = 18.5 \pm 0.5\ s^{-1}$). In the DNA solution, R_1 is smaller than both transverse relaxation rates ($R_1 = 34.2 \pm 0.7\ s^{-1}$, $R_{2s} = 39 \pm 1\ s^{-1}$, $R_{2f} = 106 \pm 5\ s^{-1}$). The lineshape analysis and the TQF experiments clearly suggest that the relaxation of ^{23}Na in the 11-basepair oligonucleotide solution is not outside the extreme narrowing condition. However, the observed significant difference between R_1 and R_2 suggests the contrary. To further investigate this apparent contradiction, measurements of the relaxation rates in the oligonucleotide solution were also performed with a 250 MHz and a 600 MHz spectrometer (^{23}Na resonance frequencies of 66.1 MHz and 158.7 MHz). If the ^{23}Na is outside the extreme narrowing condition, R_1 and R_2 should be frequency-dependent. The relaxation rates are slightly (but significantly) frequency-dependent in the oligonucleotide solution and R_1 is also smaller than R_2 at these two frequencies. However, no TQF signal is observed at these two frequencies. Based on these results, we conclude that the relaxation of ^{23}Na in the 11-basepair oligonucleotide solution is outside the extreme narrowing condition. Because R_1 is only slightly smaller than R_2 the difference between R_{2f} and R_{2s} is expected to be small and, clearly, not large enough to give rise to an observable non-Lorentzian lineshape, a biexponential transverse relaxation, or a TQF signal. Finally, it is worth mentioning that the model proposed by the groups of Halle and Leyte (Levij et al., 1981; Halle et al., 1984) for the relaxation of ^{23}Na in DNA solutions is capable of explaining the observed results for the 11-basepair oligonucleotide solution.[1]

^{23}Na-NMR study of oligomeric DNA duplex denaturation

The R_1 and R_2 relaxation rates of the ^{23}Na present in a 0.4 mM solution of the double-strand oligonucleotide were measured as a function of temperature (290–358 K) with a 360 MHz spectrometer. The longitudinal relaxation rate was measured using the inversion recovery method. The transverse relaxation rate was measured using the CPMG sequence for the small R_2 relaxation rates ($<25\ s^{-1}$) and the spin-lock experiments ($R_{1\rho} = R_2$) for the large relaxation

[1]The small difference between R_1 and R_2 could be due to the fact that there are not enough ^{23}Na ions in the vicinity of the oligonucleotide (small x_A in Eq. 1) or to the fact that the difference between the relaxation rates of the ^{23}Na in the vicinity of the oligonucleotide, $R_{1,A}$ and $R_{2,A}$, is small. A small x_A is not likely because the oligonucleotide was desalted in order to maximize the proportion of cations in the vicinity of the oligonucleotide. In the model proposed by the groups of Halle and Leyte (Levij et al., 1981; Halle et al., 1984) for the relaxation of ^{23}Na in DNA solutions, the spectral density used to describe the relaxation of ^{23}Na in the vicinity of the oligonucleotide is the sum of two components. One component is a constant and corresponds to a term in the relaxation of ^{23}Na that is inside the extreme narrowing condition. The second component is a Lorentzian and corresponds to a term in the relaxation of ^{23}Na that is outside the extreme narrowing condition. If the contribution of this second term is small (small coupling constant), the difference between $R_{1,A}$ and $R_{2,A}$ is small at all frequencies and the relaxation rates are slightly but significantly frequency-dependent.

rates. For comparison, the ^{23}Na longitudinal relaxation rate was also measured in a 0.8 mM solution of the single-strand A and in a 4.5 mM solution of NaCl. For all experiments described here, the ^{23}Na resonance lines are clearly mono-Lorentzian and single longitudinal and transverse relaxation rates are obtained. The results of the thermal denaturation experiment are shown in Fig. 2. The relaxation rates are plotted in a logarithmic scale as a function of the inverse of the temperature.

The variation of $\ln(R_1)$ as a function of the inverse of the temperature is essentially linear for NaCl (a deviation is only observed at $T > 330$ K). This is what is generally observed for NaCl when the range of temperatures under study is small (50 K) (Nordenskiöld et al., 1984). The variation of $\ln(R_1)$ is also essentially linear for the single-strand oligonucleotide but, at all temperatures, the relaxation rate is larger than for the NaCl solution. This is due to the negatively charged phosphates on the oligonucleotide backbone, which are new sources of electric field gradients. For the double-strand oligonucleotide the variations of $\ln(R_1)$ and $\ln(R_2)$ are nonlinear and similar to the variations observed in solutions of DNA (Van Dijk et al., 1987; Lematre et al., 1988; Groot et al., 1994). Below 340 K the R_2 of the double-strand oligonucleotide is always greater than R_1 and above this temperature $R_2 = R_1$. As mentioned previously, the observed difference is too small to be able to extract information on the fluctuations of electric field gradients in the vicinity of the oligonucleotide. At low temperatures, R_1 for the double-strand oligonucleotide is significantly larger than the R_1 for the single-strand oligonucleotide, while at the higher temperatures the R_1 are essentially identical. This clearly indicates that the ^{23}Na relaxation rates are sensitive to the denaturation of double-strand oligonucleotides. The reason why the relaxation rates of ^{23}Na in the double-strand oligonucleotide solution are larger than in the single-strand oligonucleotide solution could be due to 1) a larger relaxation rate for ^{23}Na in the vicinity of the double-strand oligonucleotide, or 2) a greater proportion of Na^+ ions in the vicinity of the double-strand oligoelectrolyte compared to single-strand oligoelectrolyte.

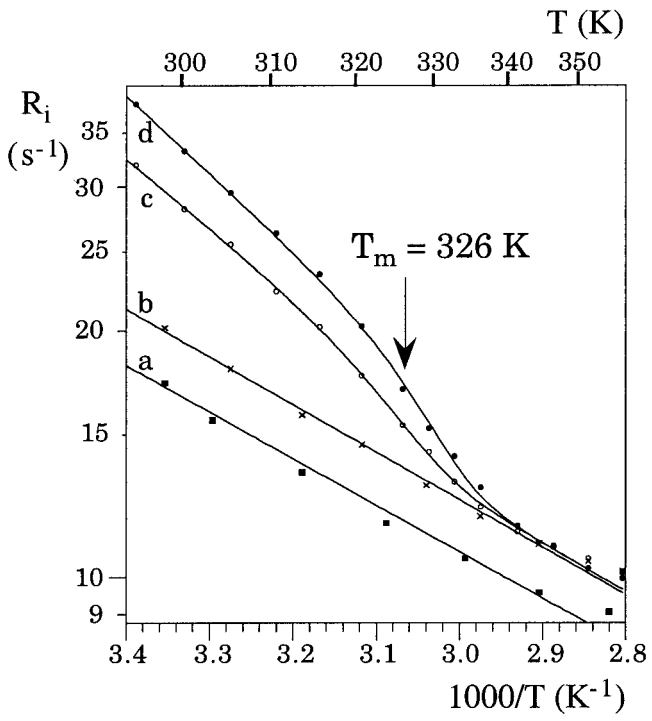

FIGURE 2 ^{23}Na relaxation rates as a function of the temperature at 95.3 MHz. *Curve a:* R_1 in a 4.5 mM NaCl solution; *curve b:* R_1 in a 0.8 mM single strand A solution; *curve c:* R_1 in a 0.4 mM solution of double-strand oligonucleotide; *curve d:* R_2 in a 0.4 mM solution of double-strand oligonucleotide.

^{23}Na-NMR melting curves have been reported in the literature for a few polymeric DNA systems (Bleam et al., 1983; Mariam and Wilson, 1983; Van Dijk et al., 1987; Lematre et al., 1988; Groot et al., 1994) but not for oligonucleotides. A melting temperature has, however, never been extracted from the reported ^{23}Na-NMR data. It should be possible to do this using the "two-state" model, which supposes that the oligonucleotide is either in the double-strand or in the single-strand form. The ^{23}Na is in fast exchange on the NMR timescale and the observed relaxation rate (R_{obs}) can be given by the following expression:

$$R_{obs} = (1 - \alpha) \cdot R_{ds} + \alpha \cdot R_{ss} \qquad (2)$$

where

- α is the fraction of strands in the single-strand form;
- R_{ds} is the relaxation rate of ^{23}Na in a solution containing only double-strand oligonucleotide and is the weighted sum of the relaxation rate of ^{23}Na in the vicinity of the double-strand oligonucleotide and the relaxation rate of ^{23}Na in the bulk (see Eq. 1);
- R_{ss} is the relaxation rate of ^{23}Na in a solution containing only single-strand oligonucleotide and is the weighted sum of the relaxation rate of ^{23}Na in the vicinity of the single-strand oligonucleotide and the relaxation rate of ^{23}Na in the bulk (see Eq. 1). The assumption is made that the proportion of ^{23}Na and the relaxation rate of ^{23}Na in the vicinity of the single-strand oligonucleotide are identical for both strands.

The variation of R_{ss} and R_{ds} with the inverse of the temperature are assumed to be exponential. This assumption is based on the experimental observation that the variations of $\ln(R_{ss})$ (Fig. 2, *curve b*) and of $\ln(R_{ds})$ for low temperatures (Fig. 2, *curve c*) are linear with the inverse of the temperature.

Using the "two-state" model, α can be expressed as a function of the change of enthalpy, ΔH^0, and entropy, ΔS^0,

associated with the denaturation process (Petersheim and Turner, 1983a):

double-strand ⇄ single-strand A + single-strand T

$$K = \frac{\alpha^2 \cdot C_0^2}{C_0 \cdot (1 - \alpha)} = \exp\left(- \frac{\Delta H^0}{R \cdot T} + \frac{\Delta S^0}{R} \right) \quad (3)$$

where C_0 is the total concentration in strand A, which is equal to the total concentration in strand T. This model assumes that ΔH^0 and ΔS^0 are independent of temperature (no heat capacity change: $\Delta C_p = 0$). Since at $T = T_m$, $\alpha =$ ½ and K = $C_0/2$, Eq. 3 can be expressed as a function of the melting temperature:

$$\frac{\alpha^2 \cdot C_0^2}{C_0 \cdot (1 - \alpha)} = \exp\left(- \frac{\Delta H^0}{R} \left(\frac{1}{T} - \frac{1}{T_m} \right) + \ln \frac{C_0}{2} \right) \quad (4)$$

An expression for α can be extracted from Eq. 4 and inserted into Eq. 2, which can then be fitted to the experimental melting curve treating ΔH^0, T_m, and the parameters describing the variation of $\ln(R_{ds})$ with temperature ($\ln(R_{ds}) = a + b/T$) as variable parameters. The parameters describing the variation of $\ln(R_{ss})$ with temperature ($\ln(R_{ss}) = c + d/T$) were extracted from the experimental data for the single-strand oligonucleotide. The melting temperature obtained in this way is $T_m = 326 \pm 3$ K ($\Delta H^0 = 280 \pm 70$ kJ/mol).

UV

UV spectroscopy is the technique most commonly used to obtain a melting temperature for oligonucleotides. In the double helix form, the base stacking induces a hypochrome effect which disappears when the bases unstack during denaturation. An increase in the absorption is therefore observed with temperature.

UV analyses are usually performed at much lower concentrations than NMR experiments (typically 10^{-6}-10^{-5} M in UV compared to 10^{-3} M in NMR). The concentration has an influence on T_m and it is therefore important to obtain a value for T_m from UV and from ^{23}Na-NMR at the same concentration. To achieve this, a spectrometer that can measure absorbances up to a value of 3 was used and the absorbance was recorded at 290 nm instead of at 260 nm, which is the maximum of absorbance. The denaturation curve obtained as a function of temperature is shown in Fig. 3. As for the ^{23}Na-NMR data, the T_m was determined using the "two-state" model (Petersheim and Turner, 1983a) where the observed molar extinction coefficient is given by:

$$\epsilon_{obs} = (1 - \alpha) \cdot \epsilon_{ds} + \alpha \cdot (\epsilon_{ss1} + \epsilon_{ss2}) \quad (5)$$

where α is the fraction of strands in the single-strand form and ϵ_{ds}, ϵ_{ss1}, and ϵ_{ss2} are the molar extinction coefficients of the double-strand and the two single-strand oligonucleotides.

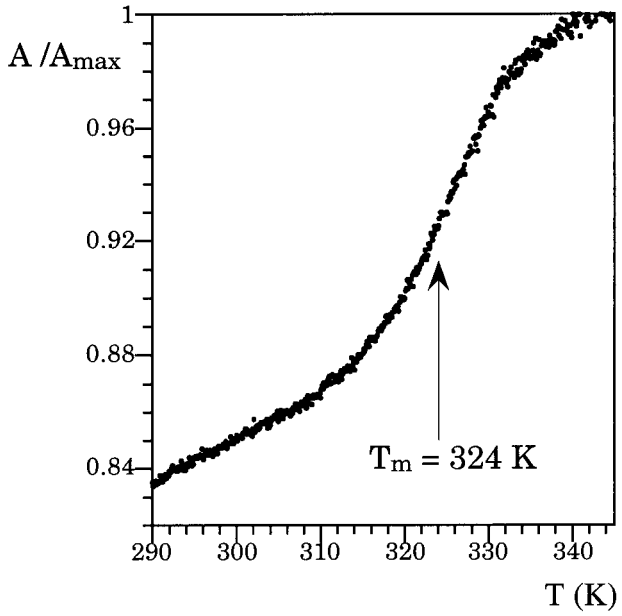

FIGURE 3 UV melting curve of the 0.4 mM solution of the 11-basepair oligonucleotide.

ϵ_{obs} can be expressed as a function of T, T_m, ΔH^0, and the parameters describing the temperature dependence of the molar extinction coefficients (which is assumed to be linear). Based on this model, the analysis of the experimental curve shown in Fig. 3 leads to $T_m = 324 \pm 2$ K ($\Delta H^0 = 290 \pm 60$ kJ/mol); a result identical, within experimental error, to the T_m value obtained from the ^{23}Na-NMR data ($T_m = 326 \pm 3$ K and $\Delta H^0 = 280 \pm 70$ kJ/mol).

^{1}H-NMR

^{1}H-NMR is extensively used to determine the 3D structure of oligonucleotides (Wemmer, 1991). It can also, as mentioned previously, be used to study the thermal denaturation of oligonucleotides. This can be done by following either the change of the chemical shift of the nonexchangeable protons (Pardi et al., 1981; Petersheim and Turner, 1983b; Braunlin and Bloomfield, 1991) or the disappearance of the signals of the imino protons (Braunlin and Bloomfield, 1988). Conformational changes associated with denaturation modify the local environment of the protons, leading to a change in their chemical shifts. The signals corresponding to the thymine methyl groups are the easiest to study because they are generally well-separated in the ^{1}H-NMR spectrum (1.3–1.8 ppm). A melting temperature, as defined previously, can then be extracted from the data. Regarding the imino protons, they are involved in the inter-strand hydrogen bonds and when the base is accessible to water, they can exchange. This leads to broadening, then disappearance of the imino signals. A disappearance temperature,

which can under certain conditions be related to the melting temperature, can be extracted from the NMR data.

Thymine methyl protons

The oligonucleotide studied in this paper contains three thymines which are situated in the central part of one of the strands (strand T). The variation with temperature of the thymine methyl signals is presented in Fig. 4. At 278 K two signals for the thymine methyls in the double-strand oligonucleotide are observed; their assignment, previously reported, is indicated in the figure (Jourdan, 1998). As the temperature increases, three new signals, whose chemical shifts change with temperature, appear. At the final temperature of 333 K a single resonance is observed at 1.67 ppm. The three new signals must correspond to the methyl groups in the single-strand oligonucleotide. This is suggested by the fact that, at 283 K, the thymine methyl groups of single-strand T exhibit resonances at 1.67, 1.60, and 1.36 ppm (data not shown) and is confirmed by the fact that the intensity of the new signals increases with temperature while the intensity of the two original signals decreases with temperature. For the whole range of temperatures, the strand pairing-unpairing process, as probed by the thymine methyls, is a slow process on the ^{1}H chemical shift timescale. A spectrum recorded at 318 K on a 360 MHz spectrometer also showed slow exchange of the signals. To our knowledge there are no reports in the literature where signals for the single- and double-strand form of the oligonucleotide are observed simultaneously during thermal denaturation. Most papers report that the thymine methyl resonances are in fast exchange (Pardi et al., 1981; Patel et al., 1982a; Tran-Dinh et al., 1982; Feigon et al., 1983; Petersheim and Turner, 1983b). However, the experimental conditions classically used to study oligonucleotides by ^{1}H-NMR are rather different from those of the present study (pH of 8.5, no buffer, oligonucleotide concentration of 0.4 mM and a sodium concentration of 8.6 mM, $[Na^+]/[P] = 1.1$).

The influence of different parameters on the exchange rate was studied. The pH in the sample was decreased to 5.9 by bubbling CO_2 in the solution. Slow exchange is still observed. The sodium concentration, at pH = 8.5, was also modified by adding NaCl. Spectra were recorded at 323, 333, and 343 K for six different sodium concentrations between 15 and 185 mM. Fast exchange is never observed, but extensively broadened signals are observed at 343 K for a sodium concentration of 185 mM, indicating that the oligonucleotide is in an intermediate exchange regime. Studies conducted in Grenoble on a 0.8 mM solution of the same oligonucleotide at a pH of 7 (phosphate buffer) and a sodium concentration of 100 mM ($[Na^+]/[P] = 6.25$) show the thymine methyls in an intermediate-to-fast exchange regime for the temperature range where both single- and double-strand oligonucleotides are present in the solution (between 338 and 358 K, M. Jourdan, unpublished results).

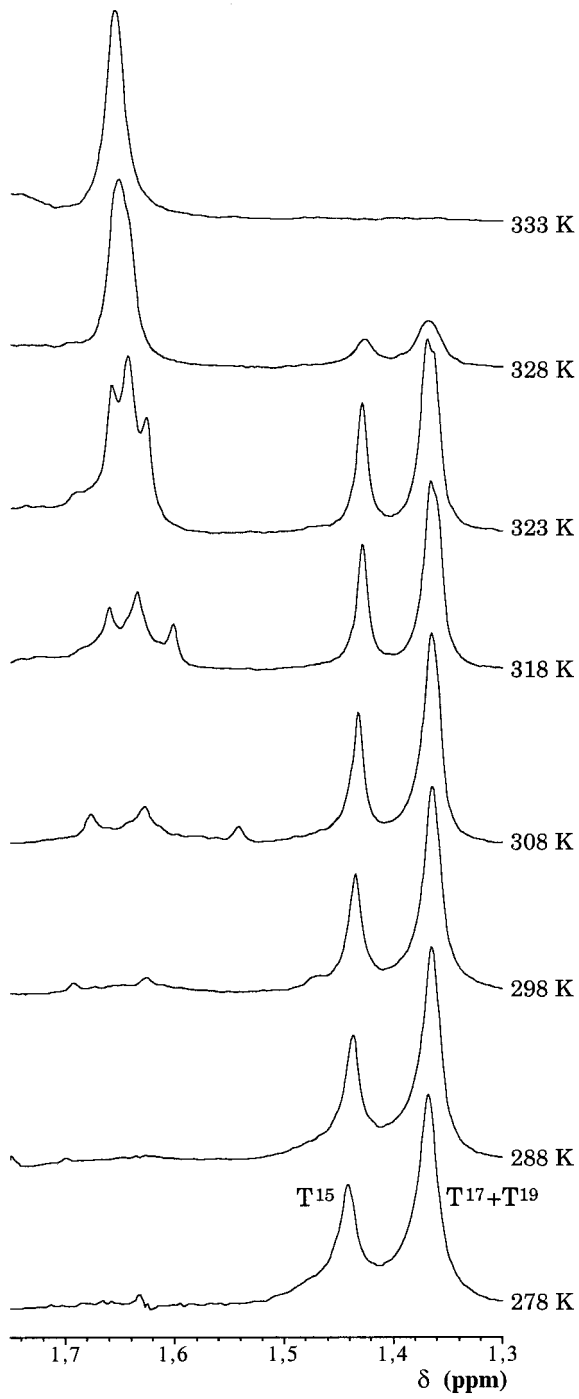

FIGURE 4 ^{1}H-NMR (600 MHz) spectra of the thymine methyls as a function of the temperature. Assignments are indicated in the figure.

The broadened resonances of the methyl groups shift progressively from the value of the resonance in the duplex to the value of the resonance in the single strand. This suggests that it is perhaps the combined effects of Na^+ concentration, pH, and oligonucleotide concentration that determine the exchange regime.

When the thymine methyl resonances are in fast exchange, a melting temperature is obtained from a plot of the observed chemical shifts as a function of temperature. These chemical shifts are the weighted average of the chemical shifts in the single-strand and in the double-strand form of the oligonucleotide. Some authors comment on the fact that they observe intermediate exchange and estimate that, in these cases, the error on T_m extracted from the variation of chemical shift can be of the order of 4–6 K (Pardi et al., 1981; Feigon et al., 1983). When the protons are in slow exchange, it is possible to obtain a value for a melting temperature from the plot of the intensity of the methyl signals of the single- and double-strand oligonucleotide as a function of temperature. For our oligonucleotide a T_m value of 321 ± 2 K was obtained in this way. This value is a little lower than those obtained via the ^{23}Na-NMR or the UV studies. However, due to the experimental errors, it is not possible to conclude that this difference is significant.

Imino protons

The spectra of the imino protons for increasing temperatures are shown in Fig. 5. The assignment of the signals has been previously reported (Jourdan, 1998) and is indicated in the figure. As the temperature increases, the signals of the imino protons disappear due to their fast exchange on the NMR timescale with solvent protons. The imino protons can exchange when they are accessible to the solvent. This occurs following denaturation or following the small-scale opening of an individual or a few basepairs (Fig. 6) (Braunlin and Bloomfield, 1988). The disappearance of the imino protons therefore depends on the rate of strand dissociation (k_d) and association ($k_a \cdot C_0$, where C_0 = [strand T] = [strand A]), on the rate of single-basepair opening (k_{op}) and closing (k_{cl}), and also on the rate of proton exchange from a single open base (k_{tr}^{I}) and from a base in an unpaired strand (k_{tr}^{II}). With the assumption that proton exchange from the unpaired strand is much faster than strand association ($k_{tr}^{II} \gg k_a C_0$), the rate of proton exchange can be given by Eq. 6 (Braunlin and Bloomfield, 1988):

$$k_{ex} = k_d + k_{op}k_{tr}^{I}/(k_{cl} + k_{tr}^{I}) \quad (6)$$

This expression shows that imino proton exchange cannot, in all cases, be directly related to denaturation.

In the 11-basepair oligonucleotide the first signals to disappear are those corresponding to the terminal guanines (G^{12} and G^{22}) (Fig. 5). At 283 K they are no longer visible. The signals of the guanines next to the terminal ones (G^{2} and G^{10}) start to broaden at 308 K and have disappeared at 323 K. The signals of all other imino protons, here below referred to as central imino protons, start broadening toward 318 K and disappear at 333 K. The data clearly indicate that fraying of the two last basepairs occurs. For increasing temperature, the decrease in the relative intensity of the central imino protons is found to be identical to the decrease observed for the nonexchangeable protons in the double-strand oligonucleotide (see, for instance, imino signals of G^{16} and G^{18} in Fig. 5 and the methyl signals of T^{15}, T^{17}, and T^{19} in Fig. 4). This observation and the fact that there is no difference in the broadening of thymine and guanine imino signals suggest that single-basepair opening does not contribute significantly to the exchange of central imino protons (if proton exchange following single-basepair opening were not negligible, then the intensity of the imino protons would decrease faster than the intensity of nonexchangeable protons. Furthermore, when proton exchange following sin-

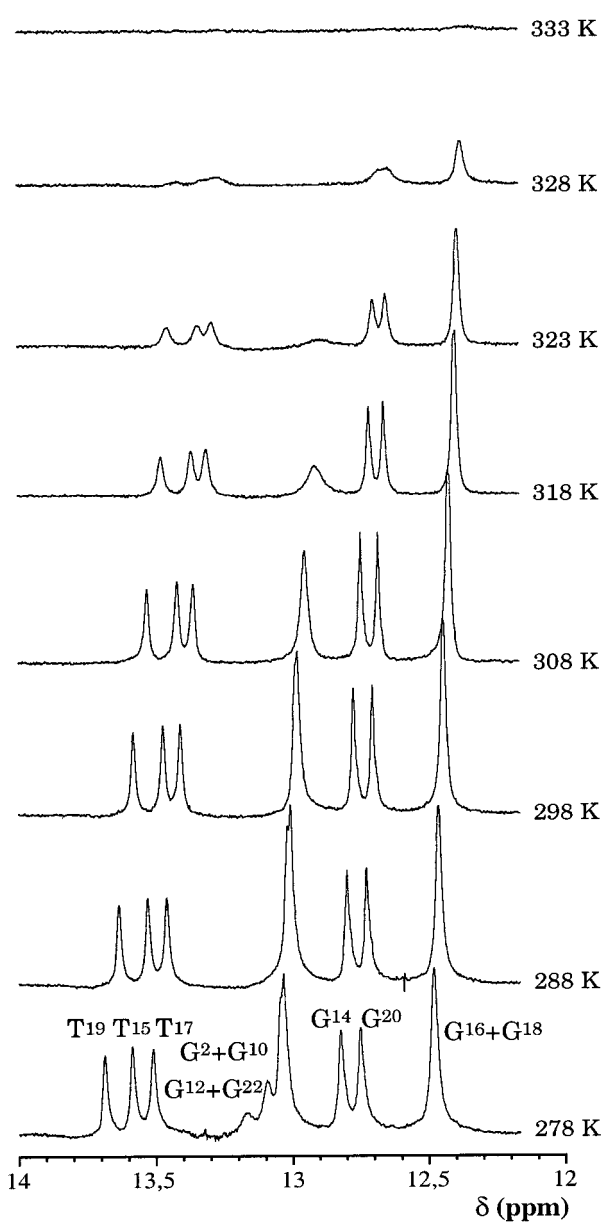

FIGURE 5 ^{1}H-NMR spectra of the exchangeable imino protons as a function of the temperature. Assignments are indicated in the figure.

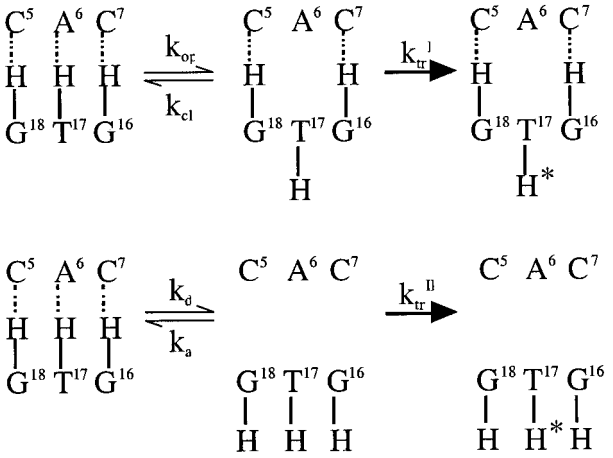

FIGURE 6 Kinetic model illustrating the two imino proton exchange pathways. The upper pathway represents exchange following small-scale opening of a single or a few basepairs of the duplex. The lower pathway represents exchange following complete dissociation of the duplex into single strands (Braunlin and Bloomfield, 1988).

gle base opening occurs, imino signals of T are known to broaden before imino signals of G, since AT basepairs are generally less stable than GC basepairs (Guéron, 1995)).

Because, in this case, proton exchange following single-basepair opening is negligible, the disappearance of the imino protons can be directly related to denaturation (the second term of Eq. 6 is negligible). The disappearance temperature of 333 K found for the central imino protons is above the melting temperature determined for this oligonucleotide by ^{23}Na-NMR, UV, and ^{1}H-NMR, and is near the temperature where no double-strand oligonucleotide is left. The fact that the imino signals are observed as long as double-strand oligonucleotide is present in the solution can be explained by 1) the absence of proton exchange following single-basepair opening, and by 2) slow strand dissociation on the NMR timescale (confirmed by the fact that resonances are observed simultaneously for the thymine methyl signals in the single and in the double-strand form).

The disappearance temperatures of imino protons reported in the literature are usually less than or equal to the melting temperatures measured by UV or by ^{1}H-NMR of nonexchangeable protons (Pardi et al., 1981; Patel et al., 1982a; Feigon et al., 1983). This means that above the disappearance temperature all the imino protons are in fast exchange even though the oligonucleotide is in the double-strand form during 50% of the time. This is what is observed for the 11-basepair oligonucleotide at a salt concentration of 185 mM: the melting temperature for the oligonucleotide is 340 K and the disappearance temperature is situated between 338 and 343 K. According to Eq. 6, the exchange is fast because strand dissociation and/or proton exchange following single-basepair opening are fast. For the 11-basepair oligonucleotide at a salt concentration of 185 mM, sequential broadening is observed: broadening of the three thymines and of G^{14} and G^{20} imino signals begins at 313 K, while broadening of the G^{16} and G^{18} signals begins at 328 K. This suggests that proton exchange following single-basepair opening is significant and, in this case, as in many cases reported in the literature, the disappearance of the imino protons cannot be directly related to denaturation.

CONCLUSIONS

^{23}Na-NMR spectroscopy has not, to date, been extensively used to obtain information on the structure and stability of DNA or oligonucleotide systems. Molecular structural parameters are generally obtained via ^{13}C, ^{31}P, ^{15}N, or ^{1}H-NMR studies. The ^{23}Na ions can, however, be considered as "spins-spies" of the biomolecule and ^{23}Na-NMR appears as a very interesting complement to the "classical" NMR methodologies because it can yield information on the ionic atmosphere and on the dynamics of the system. Indeed, the ^{23}Na relaxation can be influenced by the dynamics in the hydration shell of the ions and by the dynamics of the oligonucleotide. It can also be influenced by the movements of the counterions along the polyphosphate chain in the vicinity of the oligonucleotide and from the bulk to the vicinity of the oligonucleotide. For the 11-basepair oligonucleotide studied in this paper, the ^{23}Na relaxation is outside the extreme narrowing condition but, unfortunately, it was not possible to extract both transverse relaxation rates and ^{23}Na-NMR could not be used to obtain this quantitative dynamical information on the ionic atmosphere in the vicinity of the oligonucleotide.

^{23}Na-NMR was, however, used to study the thermal stability of the double helix structure of an 11-basepair oligonucleotide. The melting curves obtained by ^{23}Na-NMR were compared to those obtained by ^{1}H-NMR and UV spectroscopy. The UV and ^{23}Na-NMR melting curves, analyzed using the two-state model, give the same values for the melting temperature and ΔH^0. This suggests that the modifications in the ionic atmosphere, probed by ^{23}Na-NMR, and the modifications in the stacking of the bases, probed by UV, occur at the same temperature. The melting temperature determined from ^{1}H-NMR data is slightly lower but, due to the experimental errors, it is not possible to conclude that this difference is significant.

Analysis of the thymine methyl resonances and of the imino resonances in the ^{1}H-NMR spectra provided information on the dynamics of the denaturation process. Slow exchange, on the NMR timescale, is observed between the signals of thymine methyls in the single-strand and double-helix conformation. To our knowledge, slow exchange for these resonances has never been reported in the literature. This behavior could be due to the low salt content in the sample (sodium to phosphate ratio close to 1) because intermediate-to-fast exchange is observed at higher salt concentrations. The study of the disappearance of the imino protons suggests that single-basepair opening does not con-

tribute significantly to the disappearance in the desalted solution, although this process is important in solutions containing added salt. This suggests that the dynamics of the oligonucleotide during the melting process is not the same at low or high salt concentration. It also shows that it is important not to confuse melting temperature and disappearance temperature of imino protons.

We can hope that in the near future the coupling of experimental studies, like those described in this work, and molecular dynamics calculations (computer experiments) will lead to a more quantitative description of the motions of the nucleic acids and their ionic atmosphere in the vicinity of the oligonucleotide. The simulations could help to characterize the dynamics of the bases during the melting process and the kind of motions responsible for the relaxation of ^{23}Na. Nevertheless, to reach this goal it will be necessary to take into account the existence of two contradictory constraints. To obtain more experimental data from ^{23}Na-NMR, it would be better to work with a oligonucleotide for which, even at room temperature, the relaxation of ^{23}Na is clearly outside the extreme narrowing condition. This probably means a larger oligonucleotide than the one studied here. To obtain significant calculated data, it would, however, be better to work with a smaller oligonucleotide to reduce the number of degrees of freedom.

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